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Citation for published version:
Delacroix, L, Vernimmen, D, Begon, DY, Jackers, P & Winkler, R 2005, Mechanisms responsible for ERBB2 gene overexpression in human breast and non-breast cancer cells. The role of AP-2 transcription factors. in *Cancer Therapy.* pp. 365-368.

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Early version, also known as pre-print

Published In:
Cancer Therapy

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Download date: 07. May. 2023

Cancer Therapy Vol 3, 365-378, 2005

Mechanisms responsible for *ERBB2* gene overexpression in human breast and non-breast cancer cells. The role of AP-2 transcription factors

Review Article

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Key words: the *ERBB* network, *ERBB2* overexpression, transcriptional elements, Molecular mechanisms, breast cancers, rodent *Neu* promoter, Distant regulatory regions, endogenous *ERBB2* gene expression, Transgenic overexpression of AP-2, mice mammary gland

Abbreviations: AP-2 binding sites, (AP2BS); chromatin immunoprecipitation, (ChIP); ductal carcinoma *in situ*, (DCIS); ETS binding site, (EBS); immunohistochemistry, (IHC); initiator like region, (Inr); invasive carcinoma, (IC)

Received: 21 June 2005; Accepted: 28 June 2005; electronically published: July 2005

Summary

The *ERBB2* gene codes for p185^{erbB2}, a transmembrane protein with intrinsic tyrosine kinase activity. P185^{erbB2} is a member of the EGFR family of growth factor receptors. The gene is expressed in embryonic and adult cells and its function is necessary throughout the entire life. *ERBB2* is overexpressed in a significant proportion of human breast cancers where it is correlated to poor prognosis for the patient. The gene is also overexpressed in non-breast cancers but researchers disagree on the prognostic significance of the overexpression in these cancers. Gene amplification and increased transcription rates account for the very high levels of p185^{erbB2} accumulated in breast cancer cells. In a proportion of breast cancer cells a moderate increase in p185^{erbB2} level is due to transcriptional deregulation alone. The mechanisms responsible for *ERBB2* gene overexpression in non-breast cancer cells is not well understood. The molecular mechanisms responsible for *ERBB2* gene overexpression have been investigated mostly in breast cancers. In this paper we review the data from the literature and our own results on the involvement of the AP-2 transcription factors family in *ERBB2* gene overexpression in breast cancer cells. We conclude that AP-2 family of transcription factors contribute to the *ERBB2* overexpression in a fraction of breast cancers. In contrast, AP-2 factors are not responsible for increased *ERBB2* expression in the non-breast cancer cells we have analyzed.

I. Introduction: the *ERBB* network

The *ERBB2* gene (also known as *HER2* or *Neu*) encodes a 185 kDa transmembrane protein, p185^{erbB2}, with intrinsic tyrosine kinase activity. P185^{erbB2} belongs to the EGF receptor (ErbB1) family of tyrosine kinase receptors, along with the products of *EGFR*, *ERBB3* and *ERBB4* genes. ErbB -1, -3 and -4 are recognized by more than 20 growth factors belonging to the EGF family. Ligand bound receptors form homodimers and/or heterodimers composed of two different receptors of the EGFR family. No soluble growth factor recognizing p185^{erbB2} with high affinity has been identified so far and p185^{erbB2} is thus considered as an "orphan receptor". The enzymatic activity of the *ERBB3* gene product is impaired by mutation in the tyrosine kinase domain. P185^{erbB2} and the *ERBB3* gene product are activated by hetero-dimerisation with another active ligand-bound receptor (reviewed in Brennan et al, 2000; Harari and Yarden, 2000; Olayioye et al, 2000; Yarden and Sliwkowski, 2001; Citri et al, 2003; Casalini et al, 2004).

The ErbB receptors together with the EGF family of ligands form the ErbB signaling network. In the healthy tissues the ligands are secreted by stromal cells and bind receptors present on the surface of epithelial cells (Burden and Yarden, 1997).

Once activated, tyrosine residues at the carboxyl-end of the receptors are phosphorylated, creating docking sites for cytoplasmic signalling molecules. This triggers several signalling cascades, resulting in differentiation, survival, migration, depending on the growth factor, the composition of the dimer and the signalling molecules present in the cell.

P185^{erbB2} is the preferred dimerisation partner for the three other receptors of the ErbB family. The heterodimers containing p185^{erbB2} last longer and are more active than all the other homo- or heterodimers. High levels of p185^{erbB2}, such as those measured in cancerous cells overexpressing the gene stimulate proliferation, inhibit apoptosis, induce migration and modify the response to chemo- and hormone- therapy (reviewed in Harari and Yarden, 2000; Olayioye et al, 2000; Yarden and Sliwkowski, 2001).

Here we summarize first some data on the normal *ERBB2* gene expression and functions. The following section presents an overview of *ERBB2* gene overexpression in breast and non-breast cancers. The molecular mechanisms leading to *ERBB2* overexpression are the main topic of this paper. We present our data on *ERBB2* overexpression in breast cancer cells. We discuss our results and the data from the literature concerning the role of AP-2 transcription factors on *ERBB2* gene overexpression. Our results on the overexpression in non-breast cancers are summarised in the last section of this paper.

II. *ERBB2* expression in healthy tissues

Low levels of membranous p185^{erbB2} were detected in epithelial cells of a variety of normal human tissues such as those of the gastro-intestinal, respiratory, reproductive and urinary tracti, as well as in skin, breast and placenta (Press et al, 1990; King et al, 1992; Camp et al, 2003).

An intact ErbB signaling network is required during embryonic development and throughout the entire life of an animal. Mice embryos carrying knocked-out *ErbB2* gene died as a consequence of defects in the development of the heart and the nervous system. Animals where the *ErbB2* gene was inactivated specifically in the heart after birth developed dilated cardiomyopathy. Formation of neuromuscular synapses, development of muscle spindles, Schwann cell function and survival of motor neurons were impaired in ErbB2 deficient mice (reviewed by Garratt et al, 2003; Holbro and Hynes, 2004). *ErbB2* gene expressed in mice colon epithelial cells ensured the survival of enteric neurons and glia (Crone et al, 2003). In the inner ear, ErbB2 expressed by supporting cells ensured the survival of spiral ganglion neurons (Stankovic et al, 2004). The differentiating virgin mouse mammary glands express and activate ErbB2. The receptor drives the alveolar differentiation during pregnancy (reviewed by Troyer and Lee, 2001; Stern, 2003).

P185^{erbB2} levels are physiologically modulated in healthy tissues and in non-cancerous pathologies. *Erbb2* expression was increased in the regenerating mice intestine after small bowel resection (Falcone et al, 1999). During the maturation of the mice (Schroeder and Lee, 1998) and rat (Darcy et al, 2000) mammary gland changes in cell types expressing p185^{erbB2} and expression levels were observed. Variations in p185^{erbB2} levels during the menstrual cycle in the adult human breast were described. The protein was more abundant during the luteal than during the follicular phase of the cycle (Gompel et al, 1996).

III. *ERBB2* overexpression in cancers

Not surprisingly given the importance of the cellular processes it regulates, the ErbB signalling network plays a central role in the development of numerous human cancers. Indeed, shortly after the discovery of the *ErbB2* gene *(Neu)* as an oncogene in chemically induced rat brain cancers, Slamon and co-workers described the amplification and overexpression of the corresponding human gene in breast and ovary cancers. Moreover, the overexpression was associated with a poor prognosis (reviewed by DiGiovanna, 1999). This initial observation has been confirmed since by numerous studies and lead to the development of p185^{erbB2} targeted therapies for breast cancer (Ross et al, 2003). Interestingly, *ErbB2* overexpression was reported recently in spontaneous canine (de la Mulas et al, 2003) and feline (De Maria et al, 2005) mammary cancers. In both species this was a poor prognostic factor.

ERBB2 gene overexpression was also observed in non-breast human cancers, such as Wilm's tumours, bladder, pancreas, colon, lung and prostate cancers. The prognostic significance of *ERBB2* gene overexpression in non-breast tumours is debated (Klapper et al, 2000; Menard et al, 2001).

Twenty to forty percent of ovary cancers were reported to overexpress *ERBB2* (Kupryjanczyk et al, 2004). The overexpression was most frequent in metastatic ovary carcinoma specimen and cancer cell lines (Hellström et al, 2001). In a subset of ovarian cancers the gene was amplified but not overexpressed (Wu et al, 2004). *ERBB2* overexpression was shown to be necessary for the growth of the ovarian cancer cells (Juhl et al, 1997; Hsieh et al, 2000) and might condition the response to chemotherapy (Abuharbeid et al, 2004).

ERBB2 status in other types of human cancer is controversial, mostly because of methodological problems.

Maurer et al, (1998) reported co-expression of *ERBB2* and *ERBB3* genes in a high proportion of primary colorectal cancers. This was interesting since in breast cancers the erbB2/erbB3 dimer was shown to be the most tumorigenic (Harari and Yarden, 2000). Vadlamudi and co-workers (1999) further showed that these receptors were constitutively phosphorylated in colon cancer cells. However, other investigators detected *ERBB2* gene amplification and overexpression only in a small proportion of primary human colon cancers (Nathanson el al, 2003; Half et al, 2004).

Increased p185^{erbB2} levels were detected by IHC in ductal pancreatic adenocarcinoma (Apple et al, 1999; work cited by Hruban et al, 2000). In contrast, Koeppen et al, (2001) did not observe a significant increase in p185^{erbB2} levels in pancreatic cancers.

According to Craft et al, (1999) and to Signoretti et al. (2000) *ERBB2* expression was increased in prostate

cancer cells after androgen ablation and in hormone independent cancers. Some authors confirmed these observations (Osman et al, 2001; Shi et al, 2001). $P185^{erbB2}$ overexpression might thus contribute to progression toward androgen independence. The high $p185^{erbB2}$ levels detected in a significant proportion of circulating prostate cancer cells supported a role for the oncogene in prostate cancer progression (Ady et al, 2004; Carles et al, 2004). However, no consensus has been reached yet as to the role of *ERBB2* in the progression of hormone independent prostate cancers, since other investigators did not observe a correlation between *ERBB2* expression and hormone sensitivity (Reese et al, 2001; Savinainen et al, 2002; Calvo et al, 2003). The discrepancy between the results is probably due to methodological problems (Sanchez et al, 2002).

In summary, the involvement of *ERBB2* gene overexpression in breast and ovary cancers progression is generally accepted. Breast cancer cells contain very high amounts of $p185^{erbB2}$ as a consequence of gene amplification combined with increased transcription rates. $P185^{erbB2}$ targeted therapies are developed for the treatment of breast cancer patients with *ERBB2* overexpression. Whether *ERBB2* gene overexpression is significantly involved in non-breast tumours progression is less clear. When present, the increase in $p185^{erbB2}$ levels in most of these tumours was moderate and the methodological problems concerning the detection of *ERBB2* gene overexpression are not solved.

IV. Molecular mechanisms leading to *ERBB2* overexpression in breast cancers

The mechanisms responsible for *ERBB2* gene overexpression were investigated almost exclusively in breast cancers. Gene amplification and increased transcription rates lead to the very high increase in erbB2 transcript and protein levels in breast cancers. Moderate overexpression was often the consequence of transcriptional deregulation alone (Jimenez et al, 2000; Pauletti et al, 2000; Ménard et al, 2001; Hammock et al, 2003; Merkelbach-Bruse et al, 2003; Owens et al, 2004). We did show by run-on experiments, increased transcription rates in breast cancer cells overexpressing *ERBB2* (Pasleau et al, 1993).

Several teams, including our own, are interested in unravelling the mechanisms of deregulated *ERBB2* transcription in breast cancer cells.

Briefly, transcription rates are controlled by binding of transcription factors to specific enhancer sequences on the promoter. Transcription factors bound to regulatory sequences (enhancers or silencers) interact directly or indirectly with general transcription factors which recruit RNA polymerase II to the core promoter. Transcription rates can be increased by different mechanisms: increased levels or activity of transcription factors; mutations in the promoter creating binding sequences for a new activator or disrupting the binding site for a repressor. Recently, epigenetic mechanisms – DNA and histone methylation, histone acetylation – were involved in gene expression levels modulations.

To the best of our knowledge no mutations of the *ERBB2* promoter have been reported in the cancerous cells overexpressing the gene. In contrast, overexpression and activation of transcription factors have been involved in *ERBB2* overexpression in breast cancer cells.

We have analyzed the transcriptional activity of a 6kb fragment of the *ERBB2* promoter. After describing the general transcriptional elements on the promoter, we focus on our data and the results from the literature concerning the role of increased levels of AP-2 transcription factors on *ERBB2* overexpression.

A. General transcriptional elements on the human *ERBB2* promoter

Three independent transcription start sites have been mapped on the *ERBB2* gene promoter (**Figure 1A**). First, a TATA (-22 to -26) and a CAAT (-71 to -75) box direct transcription initiation at the site marked +1. This site will be referred to as the main transcription start site. An initiator like region (Inr), consisting of six GGA polypurine / polypyrimidine repeats, located from -65 to -45 base pairs upstream of the major transcription start site directs a second set of transcription start sites. The TATA box and the Inr independently govern the initiation of transcription (Mizuguchi et al, 1994). A third set of transcription initiation sites has been identified recently 12kb upstream from the main transcription start site. The mRNA initiated at this upstream site was present in low amounts in all the tested cells. It has an original 5' untranslated region and encodes a protein that is identical to the one translated from the major transcripts (Nezu et al, 1999).

The polypurine (GGA)-polypyrimidine (TTC) rich region forms an internal triplex structure (H-DNA) that represses *ERBB2* gene transcription (Scott et al, 2000).

An approximately 500bp sequence at the 5' end of the 6kb fragment contains multiple AA, TA and CA dinucleotide repeats. These features are characteristic of DNA sequences associated with the nuclear matrix and mediate the attachment of chromatin loops to the nuclear matrix (Laemmli et al, 1992). These Matrix Attachment Regions (MAR) have been involved in important cellular processes such as transcription activation (Bode et al, 2000) or insulation of genes from position effects (Allen et al, 2000).

We analyzed the sequence of a 20kb fragment of the human *ERBB2* promoter with the MAR-finder program (Singh et al, 1997) to find out if the region containing the repeats has indeed the characteristics of a MAR. A maximal MAR potential was revealed in the region located between the positions -6/-5.6kb upstream from the main transcription start site (**Figure 1B**). This region of the *ERBB2* promoter could thus be implicated in the regulation of *ERBB2* transcription by organizing the chromatin domain containing the gene. Interestingly, a DNase hypersensitive site has been previously localized around position -5.5kb, indicating that this chromatin region is accessible to DNA binding factors (Vernimmen, unpublished). The accessibility is essential for MAR sequences that often co-localize with DNaseI hypersensitive sites (Bode et al, 2000).

B. Regulated transcriptional elements of the *ERBB2* promoter

1. The proximal promoter

The transcriptional activity of the proximal 500bp of the *ERBB2* promoter was in good agreement with the level of expression of the endogenous gene in different breast cancer cell lines (Hollywood and Hurst, 1993; Grooteclaes et al, 1994; Scott et al, 1994).

Three binding sites for transcription factors implicated in the overexpression of the gene have been localized on the proximal promoter. An ETS binding site (EBS) was located immediately upstream the TATA box (Scott et al, 2000) and two AP-2 binding sites (AP2BS) were located 213 (Bosher et al, 1996) and 495bp upstream from the transcription start site (Grooteclaes et al, 1994; 1999; Vernimmen et al, 2003a) (**Figure 1C**).

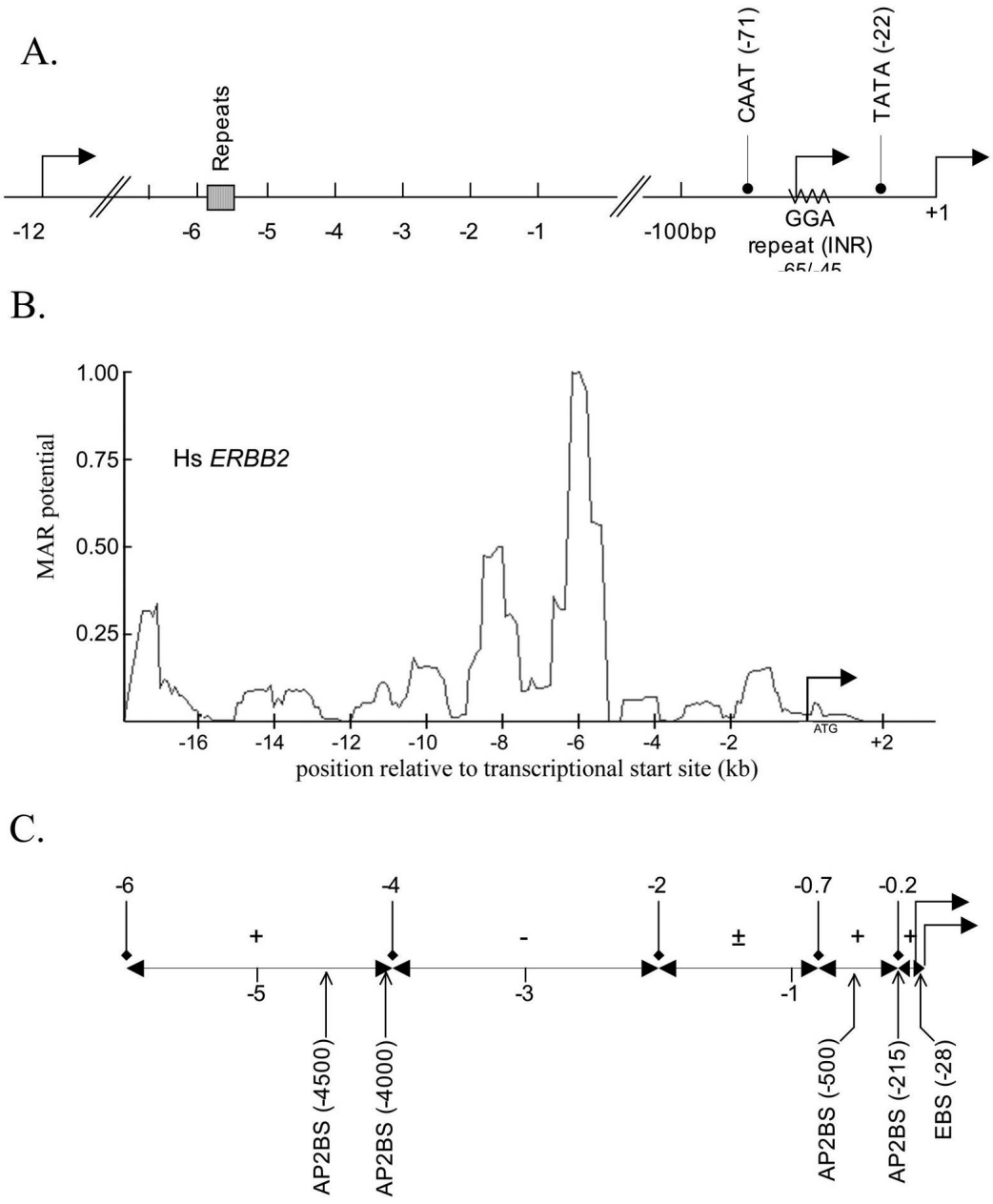

Figure 1. A. General transcriptional elements on 12kb of the human *ERBB2* promoter. The broken arrows indicate the transcription start sites. The start site at position +1 is considered as the main transcription start site of the human gene. GGA: region containing the GGA repeats corresponding to the Initiator element (Inr). The gray box indicates the region containing the dinucleotide repeats. **B**. MAR potential analysis of the *ERBB2* promoter. Positions are given relative to the major transcription start site. The sequence of an 18kb fragment of the human *ERBB2* promoter was reconstituted from Z13970 (Hudson et al, 1990), X56495 (Grooteclaes et al, 1994) and AB025285 (Nezu et al, 1999) sequences. **C**. Regulated transcriptional elements of the *ERBB2* promoter. Broken arrows indicate the transcription start sites. Positions of the EBS and AP2BS associated with *ERBB2* overexpression are indicated. The arrows point to the extremities of the promoter fragments which have been tested for activity. The plus signs indicate the promoter fragments active in the breast cancer cells which overexpress *ERBB2*. The minus signs indicate the repressing fragments. ± indicates a fragment which has different transcriptional activity according to the cell line.

The identity of the Ets transcription factor responsible for *ERBB2* overexpression is not precised yet. Several members of this vast family of transcription factors, such as ESX, are overexpressed and/or activated in breast cancer cells which overexpress *ERBB2* (Chang et al, 1997).

The AP-2 family of transcription factors includes five members: AP-2 -α, -ß, -γ (Bosher et al, 1996), -δ (Cheng et al, 2002) and -ε (Tummala et al, 2003). Breast cancer cell lines overexpressing *ERBB2* contain high amounts of AP-2 -α and -γ factors (Bosher et al, 1995; 1996; Grooteclaes et al, 1999).

We assessed the contribution of the -495 AP2BS to the promoter activity. For this purpose, we used a reporter vector containing the luciferase cDNA under the control of the proximal 750bp of the *ERBB2* promoter. New vectors were derived from the initial one where each one or both AP2BS were inactivated by site-directed mutagenesis. Mutating the AP-2 sites, either individually or in combination, reduced the activity of the promoter to one fifth the activity of the wild type promoter. Thus, both AP-2 sites must be present for full promoter activity (Vernimmen et al, 2003a).

2. Distant regulatory regions

We have investigated the transcriptional activity of promoter regions located upstream the proximal promoter (**Figure 1C**). The 3.5kb fragment preceding the proximal promoter repressed its activity in most cells. The mechanism of repression remains to be precised. The further upstream 2.2kb fragment reversed the repression specifically in breast cancer cells overexpressing *ERBB2* (Grooteclaes et al, 1994; Delacroix et al, in press).

The distal promoter region contains two AP-2 binding sites (**Figure 1C**). Binding of the factors to these sites contributed to the activity of the fragment. Indeed, the region of the distal activating fragment containing the two distal AP2BS was able to stimulate transcription from a heterologous TK promoter (Delacroix et al, in press).

Thus, the *ERBB2* promoter contains at least four AP-2 binding sites, which contribute to the overexpression of the gene in breast cancer cells (**Figure 1C**). To evaluate the contribution of AP-2 factors to the transcriptional activity of the entire 6kb fragment of the *ERBB2* promoter, we expressed an amino-terminal truncated AP-2 protein with dominant negative activity (DN-AP2). DN-AP2 has conserved the dimerisation and DNA binding domains, but lacks the transactivation domain (Williams and Tjian, 1991). BT-474 and ZR-75-1 breast cancer cells, overexpressing *ERBB2* and containing high amounts of endogenous AP-α and -γ, were co-transfected with a constant amount of a reporter vector containing the 6kb promoter fragment and increasing amounts of the DN-AP2 expression vector. We measured a dose dependent decrease in the activity of the *ERBB2* promoter, reaching half of the activity measured in the absence of the inhibitor. This shows that AP-2 factors contribute significantly to the activity of the *ERBB2* promoter (Delacroix et al, in press).

C. The rodent *Neu* promoter

The promoters of the *Neu* genes, the rat and mice orthologs of the human *ERBB2* gene, have been sequenced and the sequences were compared to those of the human promoter. The sequences of proximal promoters, extending 200bp upstream from the human main transcription start site are well conserved in the three promoters (White et al, 1992). However, there are some important differences between these sequences, indicating that the regulation of the human and the rodent genes expression might differ. For instance, the *Neu* promoters lack the TATA box and there is no initiation of transcription at the sites marked +1 on the human promoter. Moreover, some regulatory sites mapped on the rodent proximal promoters have not been found to regulate the activity of the human promoter (reviewed by Barnes and Hurst, 1997). Interestingly, as shown in **Figure 2A,** the two proximal AP-2 binding sites are not conserved in the rodent promoters.

A multalin alignment of the regions extending upstream the proximal 200bp revealed very limited sequence identity between the human, mouse and rat *ERBB2* promoters (not shown). We also analyzed the mouse and rat *Neu* gene promoter sequences with the MAR-finder program. Indeed, several experimentally identified MARs are present at similar positions in the promoters of orthologous genes (Avramova et al, 1998; Greally et al, 1999)**.** Despite limited sequence conservation, the potential of MAR occurrence on *Neu* promoters was very high for a region overlapping the position located 6kb upstream from the transcription start sites (**Figure 2B and C)**. This conserved putative MAR sequence of *ERBB2* distal promoter might thus be a general regulator of *ERBB2* gene expression. Noteworthy, the effect of such a MAR cannot be detected in reporter vectors experiments, since they are believed to act by chromatin remodeling. Transgenic animal models or stable transfection experiments have to be used to further study the contribution of the distal MAR to the expression of *ERBB2* gene.

V. AP-2 transcription factors and *ERBB2* expression in breast cancer cells

AP-2 factors activate the *ERBB2* promoter in reporter vectors. These results initiated new research to find out if these transcription factors do indeed play a role in the overexpression of the endogenous *ERBB2* gene in breast cancer cells. Three methodologies were used to address this question. First, AP-2 binding to the endogenous *ERBB2* promoter and the consequences of AP-2 down-regulation on *ERBB2* expression were analyzed in cultured cells. Second, AP-2 and $p185^{erbB2}$ levels, visualized by immunohistochemistry (IHC) on primary breast cancer sections, were compared. Third, *Neu* expression levels were investigated in transgenic mice overexpressing AP-2 in the mammary gland.

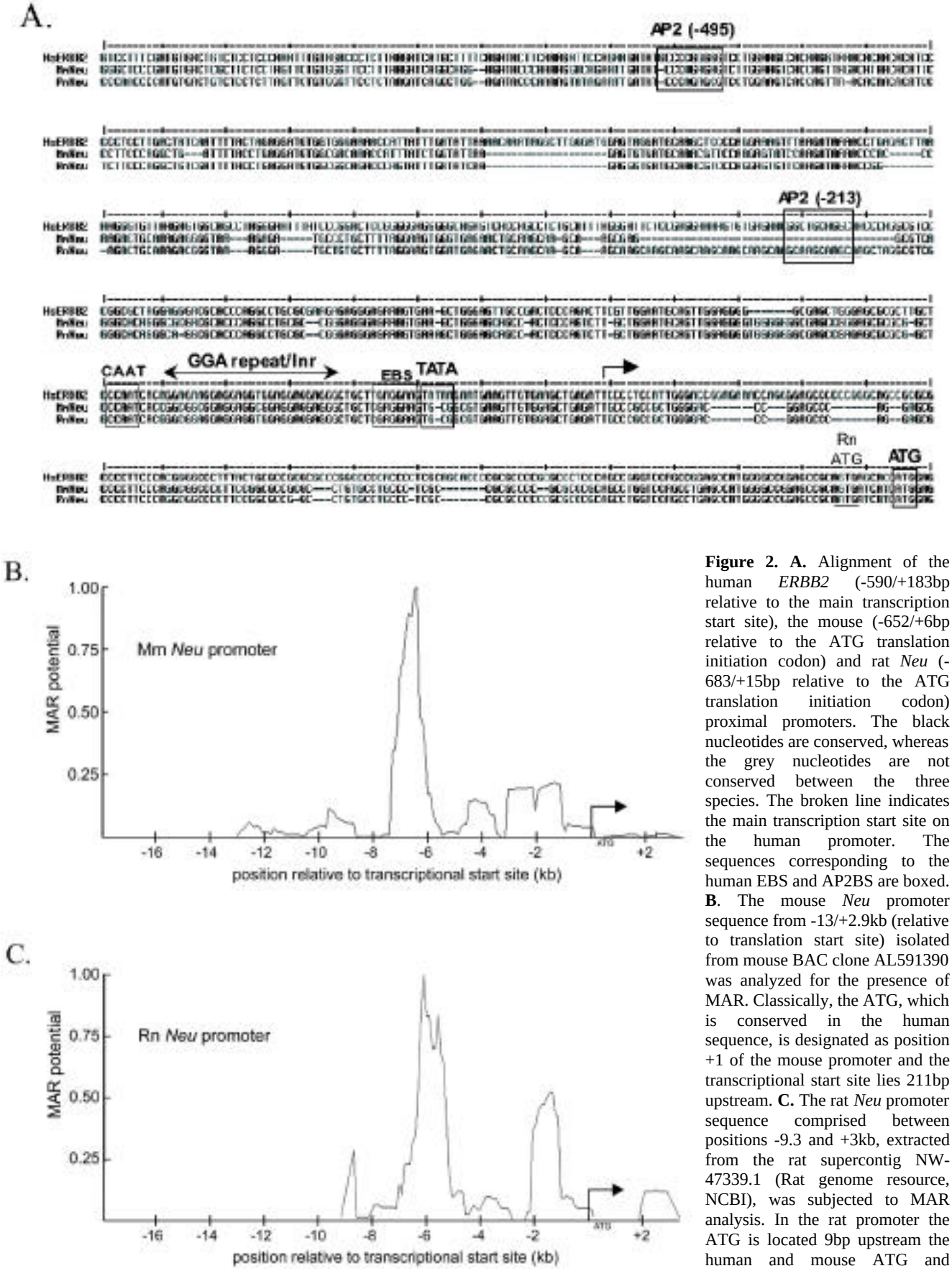

Figure 2. A. Alignment of the human *ERBB2* (-590/+183bp relative to the main transcription start site), the mouse (-652/+6bp relative to the ATG translation initiation codon) and rat *Neu* (-683/+15bp relative to the ATG translation initiation codon) proximal promoters. The black nucleotides are conserved, whereas the grey nucleotides are not conserved between the three species. The broken line indicates the main transcription start site on the human promoter. The sequences corresponding to the human EBS and AP2BS are boxed. **B**. The mouse *Neu* promoter sequence from -13/+2.9kb (relative to translation start site) isolated from mouse BAC clone AL591390 was analyzed for the presence of MAR. Classically, the ATG, which is conserved in the human sequence, is designated as position +1 of the mouse promoter and the transcriptional start site lies 211bp upstream. **C.** The rat *Neu* promoter sequence comprised between positions -9.3 and +3kb, extracted from the rat supercontig NW-47339.1 (Rat genome resource, NCBI), was subjected to MAR analysis. In the rat promoter the ATG is located 9bp upstream the human and mouse ATG and defines classically the position +1. The transcriptional start site lies at position - 203bp.

A. Modulation of the endogenous *ERBB2* gene expression by AP-2 in breast cancer cell lines

Binding of a transcription factor to the endogenous chromatin embedded promoter region is the *sine-qua-non* requirement for its activity. We thus checked for AP2 association to breast cancer cells endogenous *ERBB2* promoter by chromatin immunoprecipitation (ChIP). The chromatin from BT-474 cells was cross-linked, sonicated and immunoprecipitated with an AP-2 specific antibody. The AP-2 bound DNA fragments were PCR amplified with primers amplifying the region containing the proximal and the two distant AP-2 binding sites. The results show that AP-2 factors were bound to the proximal and the distal AP2BS (Begon et al, in press; Delacroix et al, in press). Thus, AP-2 factors are associated to the chromatin on the *ERBB2* gene promoter in the cells expressing the gene. However, association to the promoter is not sufficient to prove that the factor is active.

To prove that AP-2 factors do contribute to *ERBB2* overexpression, we measured ErbB2 mRNA levels in breast cancer cells where AP-2 -α and -γ were down-regulated by siRNA. BT-474 breast cancer cells, which overexpress *ERBB2*, were transfected with AP-2α and AP-2γ siRNA, independently and in combination. After two to four days of treatment, AP-2α, AP-2γ, ErbB2 and VEGF (an AP-2 target gene) mRNAs were quantitated by real-time RT-PCR. The results are presented in **Figure 3**. AP-2α siRNA induced a rapid down-regulation of the corresponding protein, which became undetectable 2 days after the treatment, while AP-2γ levels were unmodified. In contrast AP-2γ was greatly reduced in cells treated with the specific siRNA, without significant changes in AP-2α content. Three days after treatment with both AP-2-α and -γ siRNAs both factors became undetectable (**Figure 3A**). The evolution of ErbB2 and VEGF transcript levels was measured in the cells transfected with the AP-2 directed siRNAs. Transfection with AP-2α siRNA inhibited AP-2α mRNA but did not modify significantly either ErbB2 or VEGF transcript levels (**Figure 3B**). Comparable results were obtained in cells transfected with AP-2-γ siRNAs (**Figure 3C**). In contrast, transfection of both AP-2-α and -γ siRNAs induced a transitory but significant reduction in ErbB2 and VEGF mRNA levels (**Figure 3D**).

In conclusion, the association of AP-2 factors with the endogenous gene promoter and the inhibition of expression by the down-regulation of AP-2α and AP-2γ are strong indications that AP-2 factors do contribute to *ERBB2* overexpression in breast cancer cells. Our results further indicate that both AP-2α and AP-2γ are necessary for *ERBB2* overexpression. However, clearly other transcription factors are involved in the increased *ERBB2* gene expression observed in breast cancer cells (Delacroix et al, unpublished).

Cell lines present the advantage of being easily manipulated, however they might not reflect the properties of primary tumours. In the next sections we summarize results obtained on primary human breast cancers and in transgenic mouse models.

B. Correlation between AP-2 and $p185^{erbB2}$ levels in primary breast cancers.

A first study using two antibodies recognizing specifically AP-2α and AP-2γ reported a positive correlation between the levels of $p185^{erbB2}$ on one hand and the presence of both AP-2α and AP-2γ on the other hand (Turner et al, 1998).

A second study using a single antibody recognizing both AP-2α and AP-2β found a negative correlation between the receptor and the transcription factor levels (Gee et al, 1999).

A third report addressed the same question using a commercial anti AP-2α antibody. In only a proportion of tumours overexpressing *ERBB2* were the AP-2 levels also increased. The overall survival was shorter in patients whose tumours overexpressed $p185^{erbB2}$ but not AP-2 (Pellikainen et al, 2004). Interestingly, we observed that in *ERBB2* overexpressing MDA-MB-453 cells, AP-2 did not bind efficiently to DNA. *ERBB2* overexpression in these cells might not be dependent on AP-2 factors (Grooteclaes et al, 1999).

An additional, thorough immunohistochemical analysis localized AP-2α and AP-2γ in healthy breast tissues, in ductal carcinoma in situ (DCIS) and in invasive carcinoma (IC). Higher expression levels were detected in the healthy breast and in DCIS than in IC. Moreover, the two isoforms were expressed in distinct cell types. Glandular epithelial cells expressed AP-2α, while myoepithelial cells expressed AP-2γ. AP-2α and *ERBB2* levels were weakly but significantly correlated. In undifferentiated invasive carcinoma occasional co-expression of the two AP-2 isoforms was noted (Friedrichs et al, 2005). Interestingly, most breast cancer cell lines which overexpress *ERBB2*, overexpress both AP-2α and AP-2γ.

Finally, the relation between AP-2α gene methylation and expression was analyzed in breast cancer cells and a panel of normal breast, DCIS and IC samples (Douglas et al, 2004). AP-2α gene was unmethylated in most normal mammary epithelial cells and DCIS. AP-2α protein was detected in the nuclei of both these types of cells by IHC, with a tendency for overexpression in DCIS. In contrast, in 75% of invasive carcinoma AP-2α was hypermethylated and the protein was undetectable. These observations do not exclude an association between AP-2 and *ERBB2* overexpression during breast cancer progression. Indeed, *ERBB2* was overexpressed in a high proportion of DCIS (van de Vijver et al, 1988), while less than 30% of IC overexpress the gene.

In summary, AP-2 isoforms are expressed in healthy human breast cells, possibly in different cell types. These cells express low levels of $p185^{erbB2}$. AP-2 and *ERBB2* expression was increased in DCIS. Three out of four IHC analysis observed a tendency for a correlation AP-2 and $p185^{erbB2}$ levels in invasive carcinomas.

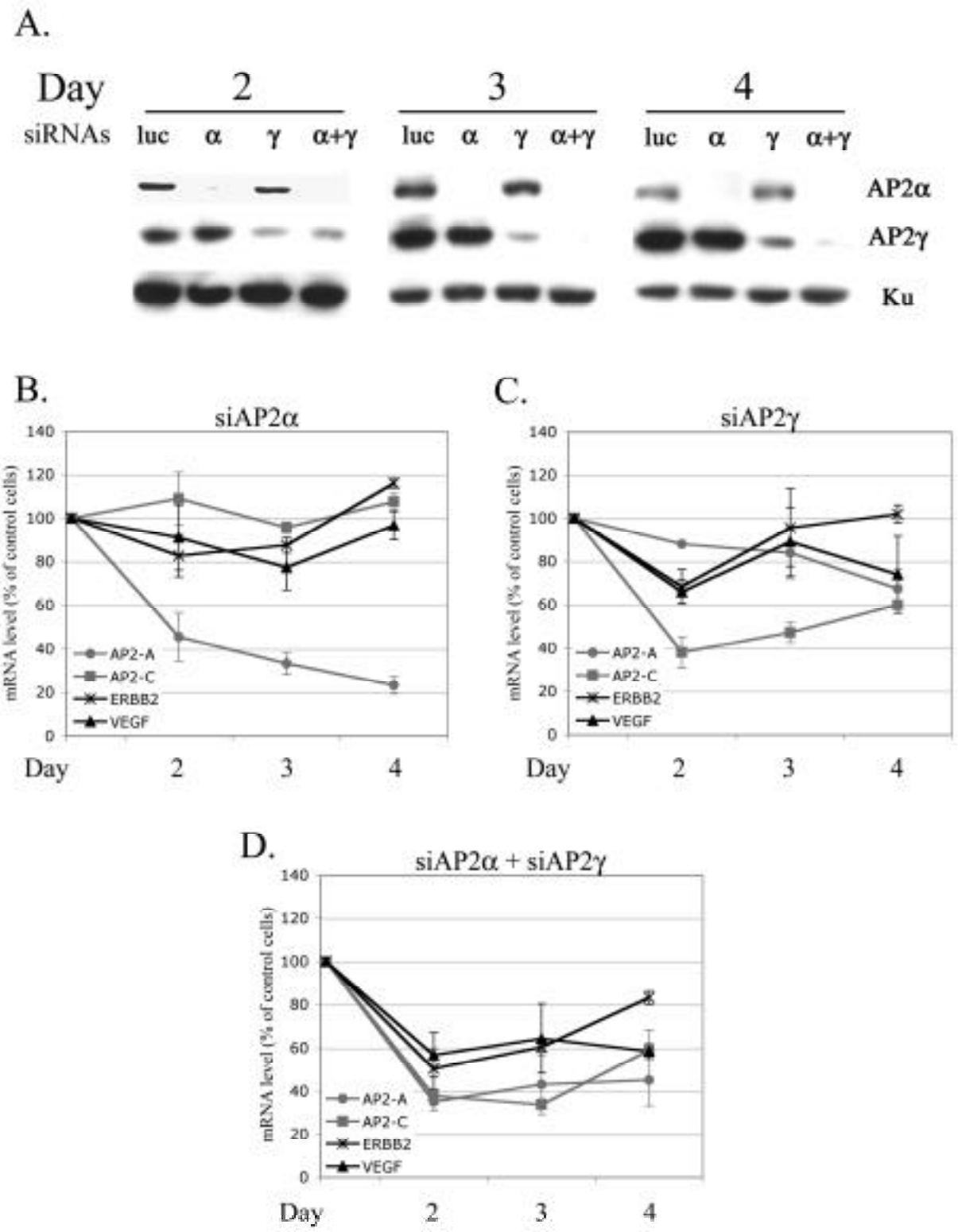

Figure 3. Suppression of AP-2α and AP-2γ expression downregulates *ERBB2* transcript level in BT-474 cells. Cells cultured in 6-well dishes were transfected on day 0 and 3 by 150nM siRNA directed against AP-2α and/ or AP-2γ transcripts. Control cells were transfected with siRNA directed against luciferase mRNA. RNA and proteins were extracted after 2, 3 or 4 days of treatment. **A.** Detection by western blotting of AP-2α and AP-2γ levels in 20mg of total proteins from cells transfected with the siRNAs. Ku protein served as control for the protein amount charged on the gel. **B.** Real time RT-PCR for AP-2α, AP-2γ, ErbB2, VEGF (AP-2 target gene) and B2M (standard gene) transcripts was performed on 1mg of total RNA from cells transfected with AP-2α siRNA. The standardized transcript levels were reported to the values obtained in control cells transfected with the luciferase siRNA. **C**. Same experiments performed on cells transfected with AP-2γ siRNA. **D**. Same experiments performed on cells transfected with AP-2α and AP-2γ siRNA.

C. Transgenic overexpression of AP-2 in mice mammary gland

Mammary targeted overexpression of AP-2α inhibited the development of the gland. The expression level of *Neu*, the mice *ERBB2* ortholog, was not modified by AP-2α overexpression (Zhang et al, 2003). Transgenic overexpression of AP-2γ elicited hyper-proliferation of the epithelial cells, which was counterbalanced by increased apoptosis, the sum of these effects leading to hypoplesia of the gland. In these mice the transgene did induce a slight increase in *Neu* expression (Jäger et al, 2003).

However, the failure of AP-2 to stimulate *Neu* gene expression in mice mammary gland does not imply that the human *ERBB2* is not regulated by these transcription factors. Indeed, as we have mentioned above, the AP2BS are missing from the mice *Neu* promoter (**Figure 2**).

The results of the experiments on the role of AP-2 transcription factors on *ERBB2* gene overexpression in breast cancer cells are summarized in **Table 1**. In breast cancer cell lines AP-2 factors stimulate *ERBB2* promoter activity. The factors are bound to the endogenous *ERBB2* promoter and their down regulation inhibits the expression of the endogenous gene. Thus, in breast cancer cell lines, experimental evidence clearly indicates that AP-2 factors stimulate *ERBB2* gene transcription. With one exception, a positive correlation was also reported between AP-2 and $p185^{erbB2}$ levels in primary breast cancers. As discussed above, the null effect of AP-2 overexpression on *Neu* expression in transgenic mice was probably due to the absence of AP2BS in the rodent promoter. These results indicate that AP-2 factors do contribute to *ERBB2* gene overexpression in some human breast cancers.

VI. *ERBB2* overexpression in non-breast cancer cells

The transcriptional mechanisms responsible for the increased *ERBB2* expression in cancers others than the breast are poorly understood. We decided to use our knowledge on breast cancer cells to understand the mechanisms leading to *ERBB2* gene overexpression in non-breast human cancer cells. We used three prostate, two ovary, five colon and seven pancreas cancer cell lines. To start, we compared the transcript and protein levels with the gene copy number. Next, we compared ErbB2 transcript and protein levels with those of AP-2 protein levels and DNA binding. Finally, we analyzed the activity of the *ERBB2* promoter fragments which have been previously characterized in breast cancers (Vernimmen et al, 2003b).

We compared *ERBB2* gene copy number, mRNA and protein levels in the non-breast cancer cells with those of well characterised breast cancer cells. *ERBB2* gene amplification was detected only in SKOV-3 ovary cancer cells, where the amplification has been described previously (King et al, 1992). These cells contained the highest amounts of ErbB2 transcripts, comparable with those measured in breast cancer cells with a similar degree of gene amplification. Variable amounts of ErbB2 mRNA were detected in the other cells we have analyzed. The ErbB2 transcript levels in one colon cancer (COLO320) and in HepG2 liver cancer cells were comparable to those of ZR-75-1 breast cancer cells, which overexpress *ERBB2* with a normal diploid set of genes.

The expression levels differed significantly between cells derived from the same cancer type (**Table 2**). So, ErbB2 mRNA levels were about seven times higher in LNCaP than in PC-3 prostate cancer cells. The difference between ErbB2 mRNA content in COLO320 and HTm29 colon cancer cells was thirteen fold. Pancreas cancer cells presented a 40-fold difference between the cells expressing the highest and the lowest amounts of the transcripts.

$P185^{erbB2}$ was detected by western blot in the majority of the analyzed cells. Protein and mRNA levels were in good agreement in the non-breast cancer cells, with the exception of colon cancer cells (**Table 2**) (Vernimmen et al, 2003b).

Next we compared AP-2 and $p185^{erbB2}$ levels in the non-breast cancer cell lines to find out if the overexpression of the transcription factors contributes to *ERBB2* overexpression. Contrary to breast cancer cells, AP-2 and $p185^{erbB2}$ levels were not correlated in the non-breast cancer cells. Strikingly, HepG2 cells expressed fair amounts of erbB2 mRNA cells but were devoid of AP-2 (Vernimmen et al, 2003b).

In order to identify the factor(s) responsible for the increased *ERBB2* expression in non-breast cancer cells, we compared the activity of *ERBB2* promoter fragments in couples of cells of the same origin expressing low and high levels of the transcript. This approach lead to the identification of AP-2 and ETS factors involvement in *ERBB2* overexpression in breast cancer cells. We could carry out these experiments only in ovary and colon cancer cells, because of very low transfection efficiencies of the other cell types. **Figures 4 B-E** summarize our results (Vernimmen et al, 2003b). The differences in promoter fragments activities between breast and non-breast cancer cells are striking. In *ERBB2* overexpressing breast cancer cells, the activity of the promoter fragments from vectors 2 and 4 was maximal, when compared to minimal promoter from vector 1. This activating potential is specific to *ERBB2* overexpression since the activity of all four promoter fragments were similar in breast cancer cell lines expressing low levels of the gene. Thus, the activating potential of promoter fragments contained in vectors 2 and 4 reflect the endogenous *ERBB2* expression level in breast cancer cells (**Figure 4B**). In contrast, all promoter fragments displayed comparable activities in HCT116 (**Figure 4C**) and in COLO320 cells (**Figure 4D**), in spite of the 3 fold difference in their ErbB2 mRNA content. In the ovary cancer cells the activity of the 6kb promoter fragment was higher in OVCAR-3 **(Figure 4E**) than in SKOV-3 cells (**Figure 4F)**, while only the latter overexpress *ERBB2*.

The low AP-2 levels might explain the low activity of the p716-LUC vector in colon and ovary cancer cells since AP-2 binding drives the activity of this fragment. These results indicate that different mechanisms lead to ErbB2 mRNA upregulation in cancerous cells of different origins.

The promoter fragments we have analyzed do not contain the sequences responsible for increased *ERBB2* gene expression in colon and ovary cancer cells. It is possible that the transcription factors responsible for the differences in transcription levels recognize sequences outside the fragments we have studied.

Another possibility is that, contrary to breast cancer cells, post-transcriptional mechanisms might be responsible for the increased ErbB2 mRNA and protein levels in the ovary and colon cancer cells we have analyzed. Indeed SKOV-3 ovary carcinoma cells express a variant ErbB2 mRNA with an extended half-life (Doherty et al, 1999). These mechanisms will have to be taken into account for the understanding of *ERBB2* gene expression regulation in non-breast cancer cells.

VII. Conclusions

P185^{erbB2} contributes to mammary carcinogenesis if present in very high amounts, reached by the combination of gene amplification and increased transcription rates. In other tumours, increased protein levels, in the highest range of the levels measured in breast cancers without gene amplification, might be sufficient for cancer progression, probably in cooperation with other oncogenic signalling pathways. Our results indicate that different mechanisms are responsible for increased receptor content in non-breast cancer cells and in breast cancer cells. These mechanisms are not known at present.

Recent data indicate that non-breast cancer cells might become resistant to chemotherapy by upregulating *ERBB2* expression. Thus, understanding the mechanisms responsible for the increase in p185^{erbB2} levels in different cancer cells is important for the development of more efficient therapeutic strategies for cancerous and non cancerous diseases involving this protein.

Table 1. Summary of the data relating AP-2 transcription factors to *ERBB2* overexpression in breast cancers.

Experiment	**Results**	**References**
Cell lines	Increased AP-2-α and -γ levels in cells overexpressing *ERBB2*	Bosher et al, 1995
Expression		
Promoter activity	AP-2-α and -γ stimulate *ERBB2* promoter fragments containing AP-2 binding sites	Bosher et al, 1995; Grooteclaes et al, 1999; Vernimmen et al, 2003a
	Mutation of AP-2 binding sites inhibits *ERBB2* promoter activity	Bosher et al, 1995, 1996; Vernimmen et al, 2003a
	DN-AP2 inhibits *ERBB2* promoter activity	Delacroix et al, 2005
Endogenous *ERBB2* promoter	AP-2 binding endogenous promoter proven by ChIP	Begon et al, 2005 Delacroix et al, 2005
	AP-2-α and -γ directed siRNAs down-regulate *ERBB2* expression	Delacroix in preparation
Immunohistochemistry on primary human breast cancer sections		
p185^{erbB2}, AP-2α and AP-2γ	Positive correlation between AP-2α and AP-2γ levels and p185^{erbB2} levels	Tumer et al,1998
p185^{erbB2}/ AP-2α	Positive correlation in a fraction of the tumors	Pellikainen et al, 2004
p185^{erbB2} AP-2α	Week correlation between AP-2α and p185^{erbB2} levels	Friedrichs et al, 2005
p185^{erbB2} and AP-2α + AP-2γ	Negative correlation	Gee et al,1999
AP-2 overexpression in the mammary gland of transgenic mice		
AP-2α	The development of the gland is inhibited	Zhang et al, 2003
AP-2γ	Increased proliferation and apoptosis	Jäger et al, 2003

Table 2. Differences in ErbB2 mRNA and protein levels in human cancer cell lines of different origins. The transcript levels were measured by quantitative RT-PCR, while the protein levels were estimated by western blotting (adapted from Vernimmen et al, 2003b). For each cancer type two cell lines are presented, one containing the lowest the second the highest amounts of ErbB2 mRNA and protein. For each cancer type, the lowest transcript and protein amounts were considered as equal to one. The relative increase in expression was calculated by dividing the highest values by the smallest values measured in cells from the same cancer type. The asterisks indicate an underestimated value for SKOV-3 protein level, because of autoradiograph saturation.

Origin	**Ovary**		**Prostate**		**Colon**		**Pancreas**	
Cell line	Ovcar-3	SKOV-3	Low (PC-3)	High (LNCaP)	Low (HTm29)	High (COLO320)	Low (SU.86.86)	High (Capan-2)
mRNA	1	60	1	7.3	1	13	1	42.5
Protein	1	13**	1	7.5	1	0.5	1	23

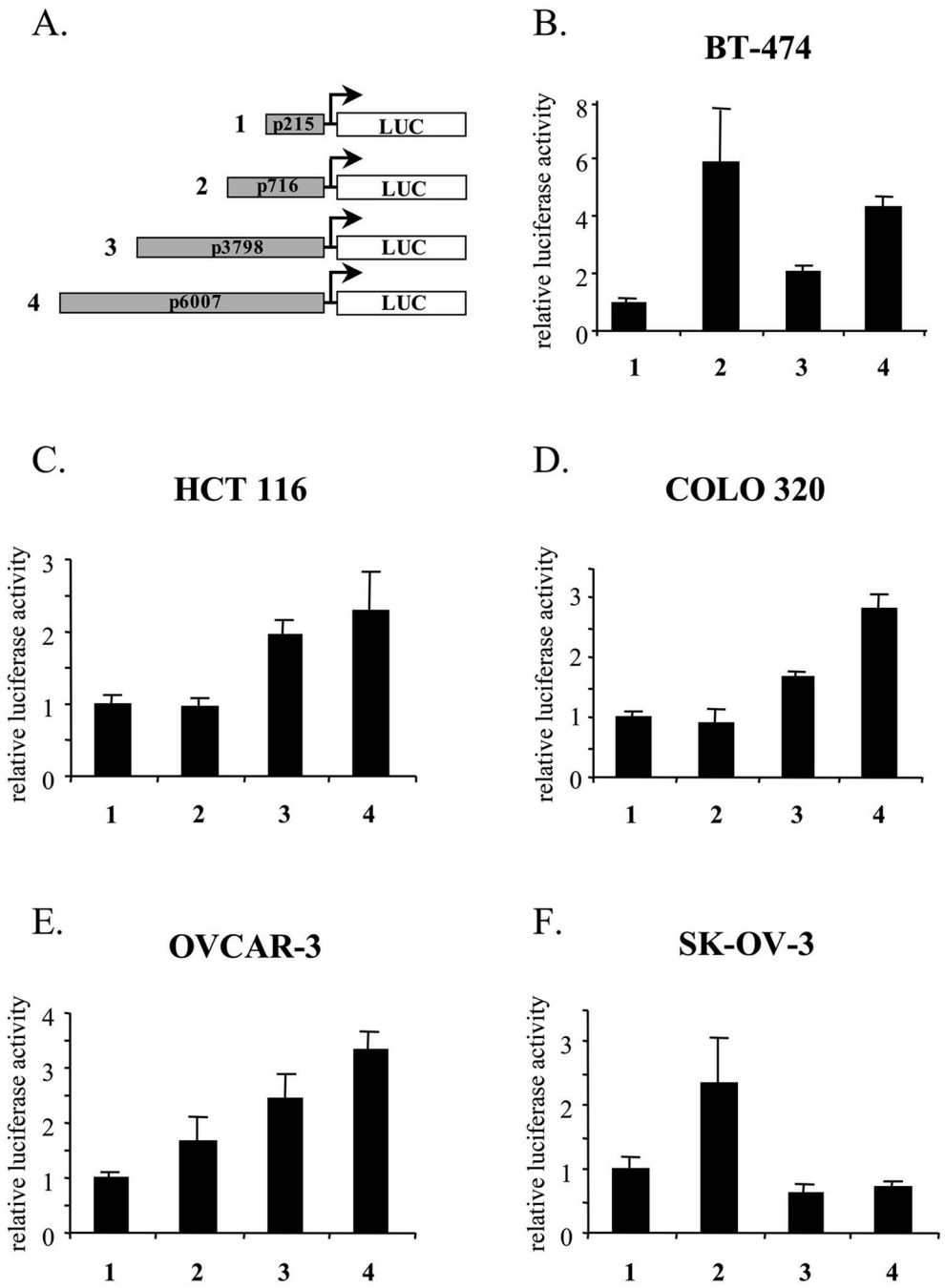

Figure 4. *ERBB2* promoter activity in human cancer cells of different origins. **A**. Reporter vectors used in this study containing the luciferase (LUC) cDNA under the transcriptional control of 215 (1), 716 (2), 3798 (3) and 6007 (4) bp fragments of the human *ERBB2* promoter. **B**. Relative luciferase activities induced by reporter vectors 2, 3 and 4 transfected into BT-474 breast cancer cells overexpressing *ERBB2*, reported to the activity induced by vector 1 considered as equal to one (Delacroix et al, in press). **C**. Relative luciferase activities induced by reporter vectors 2, 3 and 4 transfected into HCT116 colon cancer cells reported to the activity induced by vector 1 considered as equal to one. **D**. Relative luciferase activities induced by reporter vectors 2, 3 and 4 transfected into COLO 320 colon cancer cells, reported to the activity induced by vector 1 considered as equal to one. **E**. Relative luciferase activities induced by reporter vectors 2, 3 and 4 transfected into OVCAR-3 ovary cancer cells reported to the activity induced by vector 1 considered as equal to one. **F**. Relative luciferase activities induced by reporter vectors 2, 3 and 4 transfected into SKOV-3 ovary cancer cells overexpressing *ERBB2* reported to the activity induced by vector 1 considered as equal to one (Vernimmen et al, 2003b).

Aknowledgements

Our work was supported by the FNRS, the Belgian Federation against Cancer, the Centre Anticancereux près l'Université de Liège. LD and DV were recipients of Televie grants from the FNRS; BD was a recipient of FRIA fellowship and Televie grant; RW is Senior Research Associate (FNRS).

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